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Review

Xysmalobium undulatum (uzara) – review of an antidiarrhoeal traditional medicine

Ilze Vermaak, Gill M. Enslin, Thomas O. Idowu, Alvaro M. Viljoen *

Department of Pharmaceutical Sciences, Faculty of Science, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa

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ABSTRACT

Ethnopharmacological relevance: *Xysmalobium undulatum*, commonly known as uzara, is traditionally used as an antidiarrhoeal and to treat stomach cramps, dysmenorrhoea and afterbirth cramps. In addition, it was reportedly used to treat anxiety and other conditions relating to mental health.
Aim of the review: To unite the botanical aspects, ethnopharmacology, phytochemistry, biological activity, pharmacokinetic and pharmacodynamic data, toxicity and commercial aspects of the scientific literature available on uzara.
Method: An extensive review of the literature covering 1917–2014 was carried out. Electronic databases including Scopus®, Pubmed®, Google Scholar® and Google® were used to assemble the data. All abstracts, full-text articles and books written in English and German were examined and included.
Results: The phytochemistry of uzara has been comprehensively investigated and at least 18 compounds have been isolated and characterised. Uzara contains mainly cardenolide glycosides such as uzarin and xysmalorin and cardenolide aglycones such as uzarigenin and xysmalogenin. Limited scientific studies on the biological activity of uzara have been done. *In vitro* antisecretory antidiarrhoeal action was confirmed. Central nervous system activity was conflicting, *in vitro* and *in vivo* (animals) studies were inconclusive and no clinical studies have been performed. No antimutagenic effects have been reported and no toxicity up to date has been associated with uzara consumption. Significant cross-reactivity of uzara compounds with commercial digoxin and digitoxin assays may interfere with therapeutic drug monitoring.
Conclusions: The key traditional uses associated with uzara have been investigated *in vitro* and *in vivo* (animal), but clinical trial data is lacking.

Contents

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ACh, acetylcholine; AChE, acetylcholinesterase; ATCC, American type culture collection; AUC, area under the curve; cAMP, cyclic adenosine monophosphate; ^{13}C-DEPT, carbon-13 distortionless enhancement by polarisation transfer; C_{max}, peak plasma concentration; ^{13}C NMR, carbon-13 nuclear magnetic resonance; CNS, central nervous system; COSY, correlated spectroscopy; DAT, dopamine transporter; DLSL, digitalis-like serum levels; DMSO, dimethylsulphoxide; DMSO-d_6, deuterated dimethylsulphoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl 1,1-diphenyl-2-picrylhydrazyl; ECG, electrocardiogram; EI-MS, electron ionisation-mass spectrometry; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment-mass spectroscopy; FLMP, *N*-formyl-methionyl-leucyl-phenylalanine; GABA, gamma-amino butyric acid; HETCOR, heteronuclear correlation; ^{1}H–^{1}H COSY, hydrogen-1 correlated spectroscopy nuclear magnetic resonance; HMBC, heteronuclear multiple bond configuration; ^{1}H NMR, hydrogen-1 nuclear magnetic resonance; HPLC, high performance liquid chromatography; HR-MS, high resolution-mass spectrometry; HT-29/B6, human colon carcinoma cell line; 5-HT 3, 5-hydroxytryptophan; IC_{50}, inhibitory concentration 50%; INT, *p*-iodonitrotetrazolium violet; I_{SC}, short-circuit current; i.v., intravenous; K_D, dissociation constant; LC, least concern; MAO, monoamine oxidase; Max, maximum; MIC, minimum inhibitory concentration; Min, minimum; MS, mass spectrometry; Na^+/K^+-ATPase, sodium–potassium adenosine triphosphate; NAT, noradrenaline transporter; NOESY, nuclear overhauser effect spectroscopy; NMR, nuclear magnetic resonance; ROS, reactive oxygen species; SCS, substituent chemical shifts; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; STD, sexually transmitted disease; TLC, thin layer chromatography; $T_{½}$, half-life; TDM, therapeutic drug monitoring; t_{max}, time to reach C_{max}; TOCSY, total correlation spectroscopy; VD, venereal disease

* Corresponding author. Tel.: +27 12 382 6373; fax: +27 12 382 6243.
E-mail address: viljoenam@tut.ac.za (A.M. Viljoen).

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1. Introduction

The roots of *Xysmalobium undulatum* (L.) W.T. Aiton (Apocynaceae), also known as Uzara, have a long history of traditional use both internally and externally to treat diarrhoea, stomach cramps, colic, afterbirth cramps, headache and wounds amongst others. Uzara is one of the most widely used herbal remedies in South Africa (Schmelzer, 2011) and was identified by Van Wyk (2011) as a traditional remedy with potential for commercialisation. Uzara was initially introduced to the German pharmaceutical market in the early 1900s and it has been cultivated in South Africa since 1904 (Schulzke et al., 2011; Van Wyk, 2011). Few scientific studies to determine pharmacological or biological activity have been performed but *in vitro* antidiarrhoeal and *in vitro* and *in vivo* (animal) antidepressant-like action have been confirmed. The antidiarrhoeal mechanism of action of uzara is said to be a reduction in peristalsis through local stimulation of sympathetic neurons blocking smooth muscle cell depolarisation as well as antisecretory, while its antidepressant-like effect may be due to its affinity to the serotonin transporter (SERT) (Pedersen et al., 2008; Schulzke et al., 2011). The major compounds isolated from uzara include uzarin and its isomer *allo*uzarin as well as xysmalorin and its isomer *allo*xysmalorin. The presence of these cardenolide glycosides is responsible for digitalis-like effects on the heart when administered at high doses (Ghorbani et al., 1997). This review unites, for the first time, botanical information and scientific studies performed on uzara including phytochemistry, *in vitro* and/or animal studies, pharmacokinetic and pharmacodynamic data, toxicity and commercial aspects.

2. Method

An extensive review of the existent literature covering 1917–2014 was carried out. The information was assembled through searching electronic databases including Scopus®, Pubmed®, Google Scholar® and Google® using the key words uzara, *Xysmalobium* and *Xysmalobium undulatum*. All abstracts, full-text articles and books written in English and German were examined and included where appropriate.

3. Botanical aspects

Xysmalobium undulatum is part of the Apocynaceae family, formerly Asclepiadaceae. The genus *Xysmalobium* consists of about 40–45 species, all endemic to Africa, with about 18 species occurring in South Africa (Van Wyk and Gericke, 2000; Bester, 2009; Schmelzer, 2011). *Xysmalobium* is closely related to *Pachycarpus* and *Gomphocarpus*. The name *Xysmalobium undulatum* is derived from the Greek words *xysma* and *lobion*. *Xysma* means lint/bandage, covering or plaster while *lobion* which means pod, refers to the fruit which is covered with hairs. The Latin word *undulatum* alludes to the wavy (undulating) margins of the leaves (Bester, 2009).

3.1. Botanical description

Uzara is a robust, annual, geophytic herb of up to about 1 m in height (Fig. 1A) (Bester, 2009). It dies back in the winter and sprouts from a perennial rootstock in spring. The erect branches are thick and hairy and the fleshy carrot-like roots are brown on the outside and white on the inside with a peculiar, nauseating smell (Fig. 1B). A milky latex, which exudes when the plant is damaged, is present in all the plant parts. The hairy leaves are opposite, simple and entire, undulating and large (80–270 × 35–50 mm). They are roughly heart-shaped (blade lanceolate to oblong), almost stalkless with slightly thickened margins, and is prominently veined. The flowers produced from October to December are cream-green to yellowish-brown, borne in small rounded clusters along the stem (Fig. 1C). The tips of the flowers are recurved and covered in short stout white hairs which are diagnostic for the species (Van Wyk and Gericke, 2000; Bester, 2009; Schmelzer, 2011). The flowers attract a wide range of insects representing 18 families, but only the chafer beetle (*Atrichelaphinis tigrina*) and pompilid wasps (*Hemipepsis*) effect pollination. This is extremely important as uzara is genetically self-incompatible and is therefore completely reliant on pollinators for reproduction (Shuttleworth and Johnson, 2008). The large fruit (90–100 × 35–50 mm), an inflated follicle, is covered with long curly hairs (Fig. 1D). These hairs act as "parachutes" which aids in dispersing the seed when the ripe fruit bursts open (Bester, 2009; Schmelzer, 2011).

3.2. Geographical distribution and habitat

Uzara is distributed throughout mainly the eastern parts of southern Africa, occurring in Angola, Zambia, Malawi, Tanzania,

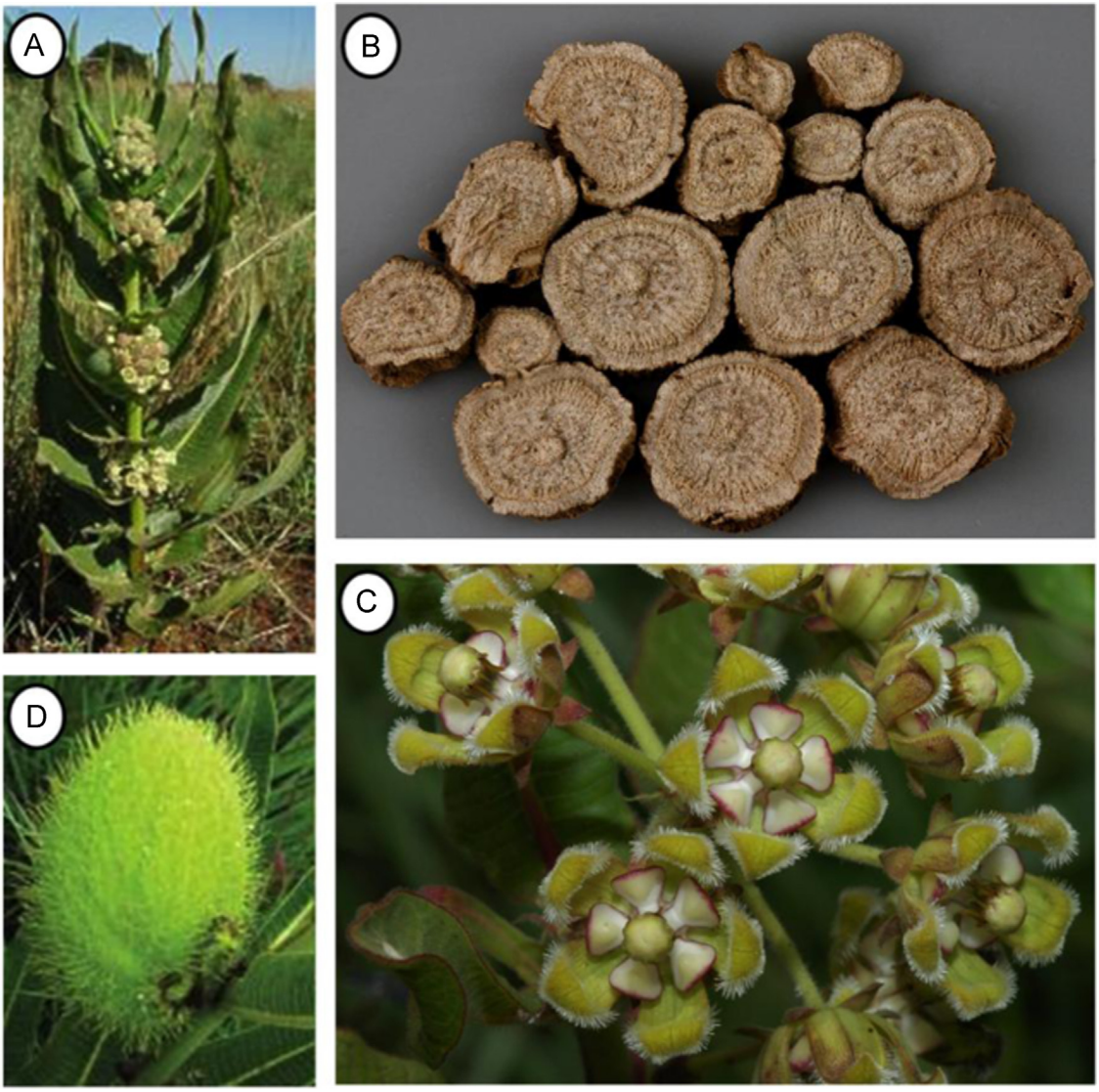


Fig. 1. Photographs of the *Xysmalobium undulatum* plant (A), dried roots (B), flowers (C) and hairy fruit (D) (Photographs courtesy of (A, D) the South African National Biodversity Institute; B. Hölcher, S. Bester; (B) A.M. Viljoen and (C) S. Johnson).

Kenya, Namibia, Botswana, Zimbabwe, Mozambique, Swaziland, Lesotho and South Africa (Fig. 2). In South Africa, it is found in the grassland biome on the verges of most roads during November and December and often in seasonally wet places in open or moist grasslands and wetlands at altitudes of 84–2000 m above sea level (Van Wyk and Gericke, 2000; Bester, 2009; Schmelzer, 2011).

4. Ethnopharmacological aspects

Uzara is regarded as one of the most important medicinal plants of South Africa as its bitter, fleshy roots have been used extensively in traditional medicine for many decades (Van Wyk and Gericke, 2000). South Africa has many official languages; therefore, uzara is known by many different common names including: English = uzara, wild bush, milkwort, wild cotton and wave-leaved *Xysmalobium*; Zulu = iShongwe, iShongwane and iShinga; Afrikaans = bitterhout, bitterwortel, bitterhoutwortel and melkbos; Xhosa = ishongwane, nwachaba and iyeza elimhlophe; and Southern Sotho = leshokoa and pohotšehla. The traditional uses in South Africa have been recorded in various publications by Watt (1935), Watt and Breyer-Brandwijk (1962), Pujol (1990), Hutchings and Van Staden (1994), Hutchings et al. (1996), Van Wyk et al. (1997), Van Wyk (2008) and Von Koenen (2001) as reported by Van Wyk and Gericke (2000) and Van Wyk (2011) and include the treatment of diarrhoea, stomach cramps, colic and afterbirth cramps as well as hysteria. Externally it is applied to abscesses and wounds (Van Wyk and Gericke, 2000; Van Wyk, 2008; Schulzke et al., 2011). The Xhosa people from the Transkei reportedly used uzara to treat hysteria and the roots are sometimes mixed with those of *Pachycarpus schinzianus* N.E.Br. to treat intestinal problems as they have the same reported medicinal uses. In addition, fomentations are applied to the chest to treat severe colds or the latex is applied to festering wounds to prevent the development of maggots. The Zulu people grind up the stem in water to be used as an emetic in poisoning while the Tswana chew a piece of the root as an antidote to food poisoning. Powdered root or a root decoction is used by the Mpondo people to treat dysentery. The Nama ingest the root as a stomach carminative and diarrhoea remedy and a cold water extract of the root has reportedly been used in the treatment of lumpy skin disease ("knoppiessiekte") in cattle (Pedersen et al., 2008; Stafford et al., 2008; Bester, 2009; Schmelzer, 2011).

Uzara is also used traditionally throughout the rest of southern Africa; as a decoction or maceration, the powdered root is used to treat intestinal problems including diarrhoea, dysentery, colic,

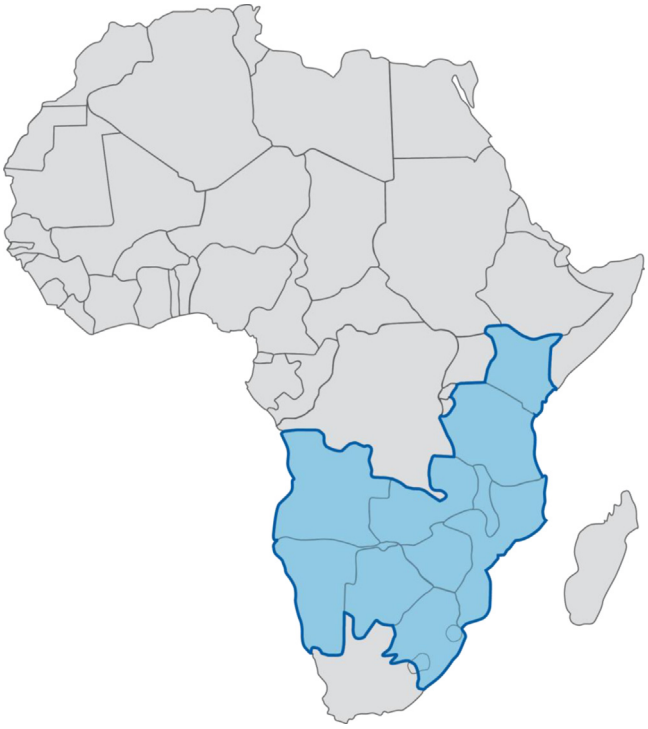
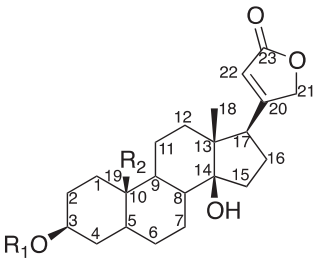
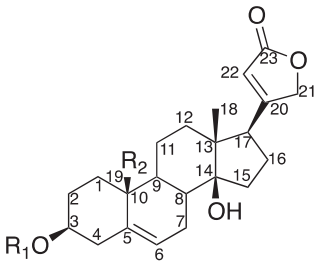


Fig. 2. Geographical distribution of *Xysmalobium undulatum*, endemic to Africa.



R1	R2	C17	compound
Genins			
H	CH_3	17αH	uzarigenin
H	CH_3	17βH	*allo*uzarigenin
H	CH_2OH	17αH	corogluacigenin
H	CH_3	16-17-epoxy	smalogenin
Glycosides[a]			
glucose-glucose[b]	CH_3	17αH	uzarin
glucose-glucose[b]	CH_3	17βH	*allo*uzarin
glucose	CH_3	17αH	desglucouzarin
glucose-glucose-glucose[c]	CH_3	17αH	uzaroside
allomethylose	CH_3	17αH	ascleposide
glucose	CH_2OH	17αH	coroglaucigenin-3-O-β-glucoside
allomethylose	CH_2OH	17αH	frugoside

Fig. 3. The genins and glycosides of uzara, 5αH (uzarigenin) series. a) Sugars conjugated with the C-3 hydroxyl of the genin *via* β-glycosidic linkage. b) Sugars conjugated *via* β-1→2-glucosidic bond. c) Sugars conjugated *via* β-1→6-glucosidic bond. (Tschesche and Brathge, 1952; Tschesche et al., 1959; Kuritzkes et al., 1963; Ghorbani et al., 1997; Pauli and Fröhlich, 2000).



R1	R2	C17	compound
Genins			
H	CH_3	17αH	xysmalogenin
H	CH_3	17βH	*allo*xysmalogenin
H	CH_2OH	17αH	pachygenol
Glycosides[a]			
glucose-glucose[b]	CH_3	17αH	xysmalorin
glucose-glucose[b]	CH_3	17βH	*allo*xysmalorin
glucose	CH3	17αH	desglucoxysmalorin
glucose	CH_2OH	17αH	pachygenol-3-O-β-glucoside

Fig. 4. The genins and glycosides of uzara, $\Delta_{5,6}$ (xysmalogenin) series. a) Sugars conjugated with the C-3 hydroxyl of the genin *via* β-glycosidic linkage. b) Sugars conjugated *via* β-1→2-glucosidic bond (Tschesche and Brathge, 1952; Tschesche et al., 1959; Kuritzkes et al., 1963; Ghorbani et al., 1997; Pauli and Fröhlich, 2000).

stomach ache and food poisoning but also menstrual and afterbirth cramps due to its antispasmodic effect. It is used to treat syphilis and urinary tract infections, coughing and heart failure and is also consumed as a tonic, diuretic and emetic. It is used as a snuff for its sedative effects and to treat headaches. The powdered root, plant decoction or latex is applied topically to treat snakebite, wounds and sores. In Namibia, the latex is applied to warts, skin rash and corns while Zambians use a root decoction to treat malaria as well as typhoid and other fevers as it causes profuse perspiration (Schmelzer, 2011).

Uzara is reportedly traditionally used not only as a medicine. The powdered root is mixed into porridge as an aphrodisiac in Zimbabwe. In South Africa the powdered root is sprinkled on hides and skins to prevent dogs from gnawing on them while they are drying out in the sun and the extremely bitter milky sap is rubbed on eggs that need to be hatched to dissuade dogs from stealing them. In addition, southern Sotho people eat the cooked young leaves as spinach or a green vegetable sometimes mixed with porridge. In southern Africa, the floss is used for stuffing mattresses and pillows. The plant is also used for its reportedly "magical" properties as a protective charm to divert storms in religious rituals, to prevent poisoning and to make dogs keen hunters (Bester, 2009; Schmelzer, 2011).

5. Phytochemistry

The phytochemistry of the genus *Xysmalobium* has evoked immense interest because of the reported occurrence of a unique cardenolide, a member of the cholestane series (A/B trans), which also reportedly differed from all other naturally occurring plant cardenolides in the spatial arrangement at C3 and C5, rendering it essentially non-cardioactive (Fieser, 1937). Of the 40–45 species reported in the literature to exist in the genus in Africa, only the chemistry of *Xysmalobium undulatum* has been extensively studied by various researchers, particularly Huber et al. (1951), Tschesche and Brathge (1952), Tschesche et al. (1955), Urscheler and Tamm (1955), Tschesche et al. (1959), Tschesche and Snatzke (1960) and Kuritzkes et al. (1963). The chemical structures of the compounds discussed in this section are shown in Figs. 3 and 4. The chief pharmacologically active constituents from uzara root include the cardenolide cardiac glycosides, uzarin (5.6%) and xysmalorin (1.5%), and their isomers *allo*uzarin (0.4%) and *allo*xysmalorin (0.1%). Their cardenolide aglycones, uzarigenin and xysmalogenin are present as minor constituents, together with *allo*uzarigenin, *allo*xysmalogenin, ascleposide, coroglaucigenin, corogluaucigenin-3-*O*-glucoside, pachygenol, pachygenol-3β-*O*-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β-sitosterol (Schmelzer, 2011).

In a definitive study in 2000, Pauli and Fröhlich used modern NMR techniques to comprehensively determine the chirality of seven uzara cardenolides. The key stereogenic centres of

uzarigenin, xysmalogenin, ascleposide, coroglaucigenin, coroglaucigenin-3-*O*-β-glucoside, pachygenol and pachygenol-3-*O*-β-glucoside were confirmed to follow regular cardiac glycoside chirality; 3βOH (i.e. non-*epi* series), 5αH (uzarigenin-type cardenolides), 10βCH_3 (A/B rings exhibiting *trans* stereochemistry), 13βCH_3 14βOH (C/D rings are *cis*) and 17αH (i.e. non-*allo* series). There has also been some uncertainty regarding the sugar portion of the major cardenolides from uzara. The glycosides were reported to be diglucosides (Tschesche and Brathge, 1952; Kuritzkes et al., 1963), but the interglycosidic linkages were the subject of some debate. In 1997, Ghorbani et al. isolated four major cardenolides in crystalline form including uzarin, *allo*uzarin, xysmalorin and *allo*xysmalorin, and reported the glycosidic linkage to be 1→2, i.e. glucopyranosyl-(1→2)-glucopyranose. This is a short summary of the phytochemistry of uzara; however, to fully appreciate the commitment of these researchers who have immensely contributed to the knowledge of uzara chemistry, it is necessary to follow the history of how the chemical structures were elucidated.

5.1. History of the identification of uzara glycosides

Xysmalobium undulatum has been the subject of phytochemical investigations since the early 1900s. Gürber reported three unidentified crystalline substances from the plant while investigating the valuable anti-diarrhoeal properties of uzara (Gürber, 1911). In 1917, Hennk investigated the chemical composition of uzara roots and identified one of these compounds as a glucoside, and named it uzarin. It was noted that the alcoholic fractions obtained during the purification of uzarin contained small amounts of a second glucoside which was amorphous and differed from uzarin in its physiological action and in its extremely bitter taste. However, it remained unidentified at that time (Hennk, 1917). In 1927, Brandwijk investigated the root chemistry with the aim of isolating the active principle(s) and establishing their chemical properties. The average water content of the dried root was determined to be 10.3%, the ash value 4.9%, total carbohydrates after hydrolysis were estimated at an average of 35.4% (expressed as glucose) and the average percentage of free sugar before hydrolysis was estimated to be 7.6% (expressed as glucose). The extract also contained very small amounts of acid-saponins, but no volatile active compounds, alkaloids or tannic acid was detected. Through repeated precipitation and recrystallisation an apparently pure glucoside, named xysmalobinum at the time, and another unnamed, apparently pure glucosidal product which deliquesced readily was isolated. After various experiments it was concluded that although the two glycosides apparently resembled each other very closely in terms of solubility (in water, alkali, acid, ether, hot alcohol), colour and colour reaction, they differed in terms of melting points, appearance under microscopic examination as well as in its biological and toxic effects (Brandwijk, 1927).

Tschesche (1933) and Tschesche et al. (1935) determined after hydrolysis experiments that uzarin consisted of a disaccharide of two glucose moieties with uzarigenin as the aglycone. In 1951, Huber and co-workers named xysmalobin from a mixture of other glycosides present in the root extract apart from uzarin. In the following year, three new minor glycoside constituents xysmalorin, urezin and uzaroside (a triglycoside of uzarigenin with the sugar comprising of only D-glucose) were isolated (Tschesche and Brathge, 1952). It was further reported in the same study that the root contained free aglycones in the form of uzarigenin, xysmalogenin and urezigenin (reportedly the 3-(α) isomer of uzarigenin), which were difficult to separate. In addition, this was the first study that made mention of xysmalogenin and xysmalorin. In 1955, Tschesche et al. identified the fourth genin in the form of an epoxide ($C_{23}H_{32}O_4$), called smalogenin. It was also in 1955 that frugoside was reportedly isolated from the seeds of uzara by Urscheler and Tamm. In 1959, Tschesche et al. isolated *allo*uzarigenin (=17α-uzarigenin) and pregnenolone in very small quantities in 1960 together with the glucoside of *allo*pregnanolone (the A/B trans derivative) and β-sitosterol. Pregnenolone is a biogenetic precursor involved in the steroidogenesis of mineralocorticoids, glucocorticoids, progestogens, androgens and estrogens (Tschesche and Snatzke, 1960). In 1963, Kuritzkes et al. confirmed that the roots were very rich in cardenolide glycosides and in addition reported for the first time the presence of coroglaucigenin, pachygenol and ascleposide (uzarigenin-β-D-*allo*methyloside). Uzarigenin and xysmalogenin in pure form was obtained through acetylation and purification *via* chromatography respectively, and it was confirmed that the seeds of the plant contain glycosides which differ in composition from the roots, notably frugoside (Kuritzkes et al., 1963).

5.2. Stereochemistry of the major uzara glycosides

Uzara was investigated by numerous researchers and it was well documented that the chief active compounds were the cardiac cardenolides and their 3-*O*-glycosides, with the major glycosides identified as uzarin and xysmalorin. Most of the reports attributed the pharmacological effects to the content of the prominent cardenolides, particularly the polar glycosides derived from uzarigenin. It had also been confirmed through acid hydrolysis, various chemical analyses and enzymatic cleavages that the sugar moieties were glucose. However, a number of challenges concerning the chemistry remained. Most of the inconsistencies and contradictions in the literature were related to the nomenclature, stereochemistry and isomerism of both the steroidal aglycone and the sugar portion of the cardenolide glycosides. Clarification was required with respect to: (i) the interglycosidic linkages in the sugar moieties; (ii) the stereochemistry of key positions, particularly the C3βOH function, 5αH (in the uzarigenin series only), 10βCH_3 (A/B rings *trans*), 13βCH_3 14βOH (C/D rings *cis*) and 17αH; and (iii) the structure assignment of the unsaturated analogues of the steroids.

The interglycosidic linkage of the sugar moieties for which no information was available prompted Ghorbani et al. (1997) to re-investigate the phytochemistry of uzara. On surveying highly reputable sources of the literature for the structure of uzarin, the researchers discovered some erroneous data from: (a) the Dictionary of Natural Products from the Chapman & Hall Chemical Database which represents this glycoside as 3-*O*[β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside] of uzarigenin; (b) Chemical Abstracts 1967: 20231-81-6 which similarly represent uzarin with the same structure; (c) Danish zoologist Nielsen in 1978 in his work entitled, "Host plant discrimination within Cruciferae…" used uzarin obtained from a pharmaceutical colleague and perpetuated this error by naming uzarin as a (1→6) diglucoside (Ghorbani et al., 1997).

Using medium pressure reverse phase column chromatography under HPLC control, four crystalline glycosides (*allo*xysmalorin, *allo*uzarin, xysmalorin and uzarin) were isolated from the root material using a commercially available alcohol extract. After enzymatic cleavage, the corresponding aglycones were identified as *allo*xysmalogenin (H-17-β-xysmalorin), *allo*uzarigenin (H-17-β-uzarigenin), xysmalogenin and uzarigenin. The sugar fractions of the hydrolysates were identified as glucose using thin layer chromatography (TLC) and the D-configuration was confirmed by optical rotation. The ^{13}C NMR spectral shifts were characteristic of the two anomeric carbons, C-1′ and C-1″. The observation of typical anomeric chemical shifts and coupling constants in the ^{1}H NMR spectra was suggestive of the presence of a biglucosyl moiety and that the two glucose units had the β-configuration at

the anomeric centres. The ^{1}H–^{1}H COSY spectra implied a (1→2) linkage of the disaccharide unit and it was confirmed by selective COSY in DMSO-d_6. HMBC experimentation further confirmed that the two glucose units are connected at (1→2) *via* a glycosidic linkage to form a β-sophorose (*O*-β-glucopyranosyl-(1→2)-β-D-glucopyranose) moiety in each of the four glycosides isolated and characterised. The interglycosidic linkage in the diglucosides was unequivocally revealed to be neither (1→4) (maltose) nor (1→6) (gentiobiose) as reported for urezin by Tschesche and Brathge (1952) and Nielsen (1978), but rather (1→2) (sophorose).

It should be noted that the modern diagnostic tools of ^{13}C-DEPT, HETCOR, ^{1}H–^{1}H COSY, NOESY, HMBC, selective COSY, EI-MS, FAB-MS and HR-MS were utilised to elucidate and confirm the structures of *allo*xysmalorin, *allo*uzarin, xysmalorin and uzarin as authentic glycosides of uzara by Ghorbani et al. (1997). ^{1}H NMR and ^{13}C NMR spectra presented typical patterns of a steroidal glycoside with a butenolide (cardenolide) ring; ^{13}C NMR suggested hydroxylation at C-14. ^{1}H NMR of *allo*xysmalorin and xysmalorin, confirmed by ^{1}H-^{13}C-HETCOR NMR correlation, supported the presence of Δ_5-unsaturation in these compounds; the C-19 methyl was confirmed by the HMBC spectra; the 17-β-cardenolide was confirmed by ^{13}C NMR highfield shifts for the 17-β-cardenolide, xysmalorin, compared to the 17-α analogue, *allo*xysmalorin. ^{1}H NMR chemical shifts were also indicative of C-17 stereochemistry, supported by NOESY correlations, which distinguish between the α and β-configurations for H-17. The differences in ^{1}H NMR and ^{13}C NMR spectra for *allo*uzarin and uzarin similarly exhibited differences in chemical shifts allowing for the distinction between the 17-α- and the 17-β epimers. FAB-MS quasi-molecular ion peak results confirmed the mass of the four glycosides and the fragment ions established the products of enzymatic hydrolysis, the aglycones, as uzarigenin and xysmalogenin. In summary, through the work of Ghorbani et al. (1997), the uncertainty and inconsistency that for a long time clouded the interglycosidic linkage of the disaccharide portion of the major uzara cardenolide glycosides was dispelled. In addition, after enzymatic cleavage of these four glycosides, and using modern NMR and MS tools, the stereochemistry of the steroid moieties was resolved to be uzarigenin, xysmalogenin, and their respective epimers, H-17β-uzarigenin (*allo*uzarigenin) and H-17β-xysmalogenin (*allo*xysmalogenin) (Ghorbani et al., 1997).

Pauli and Fröhlich (2000) noted that uzarigenin had previously been erroneously reported to bear an axial C3-OH function and had been wrongly assigned to the *allo*-series of cardenolides. Similarly, they queried the identification and correctness of the identification of urezin (Tschesche and Brathge, 1952; Tschesche et al., 1955, 1959) and its aglycone *allo*urezigenin as authentic constituents of uzara roots (Kuritzkes et al., 1963). Further to the problem of interglycosidic linkage and some stereochemical pattern confusion regarding position C-3 and C-17 which had been solved by Ghorbani et al. (1997), the relationship between the 5α-uzarigenin type (A/B rings *trans*) and the 5β-digitoxigenin-type (*cis*-fused A/B) cardenolides needed to be confirmed. In addition to this, there was also the challenge concerning the structure assignment of the unsaturated analogues of these steroids as a result of some studies on various plants belonging to the Asclepiadaceae family such as *Gomphocarpus sinaicus* Boiss. which showed double bonds at position 7 and 8 ($\Delta_{7,8}$) (Abdel-Azim et al., 1996; Pauli and Fröhlich, 2000) rather than the previously reported unsaturation at position 5 and 6 ($\Delta_{5,6}$) of the steroids (El-Askary et al., 1993; Pauli and Fröhlich, 2000). Therefore, the substituent chemical shifts (SCS) arising from the two patterns of unsaturation ($\Delta_{5,6}$ and $\Delta_{7,8}$) needed structural proof for definitive ^{1}H NMR assignments. It was this challenge and inconsistency that led to the extensive work carried out by Pauli and Fröhlich in 2000 with the aim of characterising and establishing a concrete stereochemical assignment of the key chiral positions in the uzarigenin and xysmalogenin types of uzara cardenolides and to clearly remove all contradictions surrounding the identity and the chemical structure of the uzara steroids. Pauli and Fröhlich applied a combination of the diagnostic power of selective pulses (selective TOCSY) and pulsed field gradient HMBC experiments of modern NMR techniques to deduce the complete steroid configuration and thereby unequivocally establish the stereochemistry and key chiral positions in all the uzara cardenolides as bearing regular equatorial C3-OH groups (non-*epi*-type aglycones); possessing A/B *trans* ring fusion and thus representing 5α-cardenolides (uzarigenin *trans*-fused A/B) which are diagnostically different from 5β-digitoxigenin-type (*cis*-fused A/B) cardenolides); $\Delta_{5,6}$ unsaturation for xysmalogenin and pachygenol series (verified by X-ray crystallography); possessing *cis* C/D ring fusion; and β orientation of the butenolide ring. Fig. 5 outlines the relevant sites of stereoisomerism and unsaturation elucidated by Pauli and Fröhlich. In addition, their work did not only solve the problem of stereochemical pattern, but further led to the isolation of two new cardenolides, coroglaucigenin-3-*O*-β-glucoside, and pachygenol-3-*O*-β-glucoside and five previously reported compounds including uzarigenin, asclepioside, coroglaucigenin, xysmalogenin and pachygenol (Pauli and Fröhlich, 2000).

5.3. Biosynthesis

Tschesche (1972) in his investigation into the biosynthesis of cardenolides, bufadienolides and steroid sapogenins in cardenolide-forming plants proved that pregnenolone, isolated from uzara in 1960 by Tschesche and Snatzke, is the biogenetic precursor of the cardenolides. A whole plant sample of *Digitalis lanata* Ehrh. was fed with pregnenolone glucoside labelled at C-21 with ^{14}C. After about 20 days, Tschesche discovered that 6% of the incorporated pregnenolone was found in the cardenolides, as radioactive digitoxigenin, gitoxigenin and digoxigenin were isolated after hydrolysis. It was therefore concluded that there is a close chemical relationship between the C-21 steroids, the aglycones of heart poisons with 23 carbon atoms and pregnenolone and their occurrence together with cardenolides in *Xysmalobium undulatum*. It was further shown that there was no retention of radioactivity in the remaining tetracyclic system after ozonolysis of the unsaturated lactone ring which proved that the activity was located in the lactone ring which was derived from labelled C-21 of pregnenolone. It was therefore concluded that all cardenolide-forming plants possess an enzyme system capable of transforming pregnenolone into cardenolides (Tschesche, 1972).

5.4. Structure activity relationships of the cardenolides

With respect to structural activity relationship of uzarin and other cardenolides in *Xysmalobium undulatum*, an associate Professor of Chemistry at Harvard University (L.F. Fieser) in Chapter VI (Heart Poisons) of his book titled "The Chemistry of Natural Products related to phenanthrene; 2nd edition" (1937) reported that the cardiotonic activity or general effects for cardenolides had been associated with the unsaturated lactone ring as the saturation of the ethylenic linkage in the lactone ring resulted in great decrease in toxicity as demonstrated by the digitalis-*Strophanthus* group. According to his report uzarin had been shown to exhibit no digitalis-like action (cardiotonic effect) and had been found to have about one-sixtieth the activity of ouabain (a standard tool to investigate the mode of action of cardiotonic steroids) even though it possesses the unsaturated lactone ring. The cardiotonic effect can therefore not be ascribed only to the presence of an unsaturated lactone ring, as demonstrated with uzarin. It was reported by Pauli and Fröhlich (2000) that the stereochemical constitution of

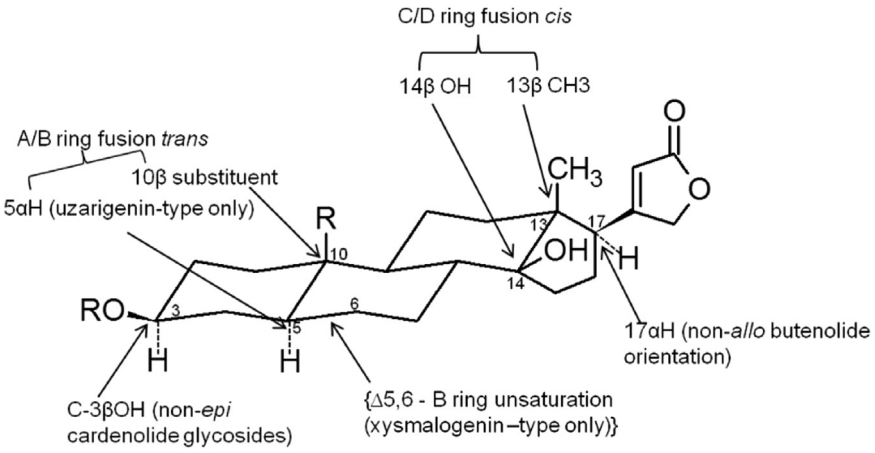


Fig. 5. The key stereogenic positions of the skeleton of uzara steroids (adapted from Pauli and Fröhlich (2000)).

seven isolated 5αH (uzarigenin series) and $\Delta_{5,6}$ (xysmalogenin series) cardenolides from uzara was compatible with the essential structure of inotropic steroids. The biological activity of the cardenolides of the uzara root comprises both cardiotropic effects and smooth muscle antispasmodic effects. The uncertainty surrounding the structure activity relationships may be in part ascribed to the numerous asymmetric centres on the steroid moiety of the molecule, resulting in multiple stereoisomers and diverse biological activity. It has therefore been argued that it is possible that the almost complete absence of cardiotonic properties in uzarin is associated with these stereochemical differences, even though it is closely related in structure to the more potent glycosides such as digitoxin. Furthermore, proof of the effect of the stereochemical pattern on activity was revealed by anhydrodigitoxigenin which probably differs from *α*- or β-anhydro-uzarigenin only in having the *epi*-configuration at C-3 and a cis linkage between rings A and B, and yet its glycoside is characterised by a high degree of potency. The report further showed the effect of stereochemical patterns on the cardiotonic effect in the case of androsterone revealing that the physiological activity drops to one-seventh the original value following an inversion at C-3, while an inversion at C-5 results in the complete loss of activity. It was also reported that the role of sugar residue appeared to be of little importance, for different glycosides of the same genin exhibits very similar in activity. Therefore, it was postulated that it is not the stereochemistry of the sugar moieties that influences the intrinsic cardiac activity of cardenolides. However, Rathore et al. (1986) indicated that sugar substituents may have a role in the binding of some glycoside stereoisomers to the Na^+/K^+-ATPase transporter, but not in others. The sugar portion of the molecule may also influence the character and intensity of the cardiac effect, by virtue of its effect on the water-solubility and the diffusibility in the system. Reports had also shown that of all the toxic cardiac cardenolides, uzarigenin is the only aglycone known to have the *A*/*B* trans configuration (Fieser, 1937). The other aglycones have been shown to have the *cis* linkage between rings A and B. This report was also confirmed by Melero et al. (2000) who reported that in digitalis-like glycosides, the steroidal framework is considered the pharmacophoric moiety, responsible for the activity of these compounds and not the sugar moieties. Specifically, the 5β, 14β-androstane-3β, 14-diol skeleton has been shown to have the same binding properties to the enzyme as digitalis compounds. In addition, the *cis* junctions between A/B and C/D rings have been reported to be an essential condition for the highest interaction energy, and therefore any change on that spatial disposition, modifying the cis to A/B trans decreases the interaction energy and therefore reduces its activity. This decrease in activity is also reportedly observed in the case of variation in configuration from 5β to 5α (Melero, et al., 2000). It is therefore not surprising then that uzara cardenolides and glycosides have no digitalis-like activity and hence little or no cardiotonic effect. In summary, it is clear that the lack of pronounced cardiotonic activity may be due in part to this divergence from the usual stereochemical pattern.

6. Biological activity

6.1. Antidiarrhoeal activity

Uzara was introduced into the market to treat acute diarrhoea and accompanying abdominal cramps in 1911 and its therapeutic effects were reported to be due to its effect on intestinal motor function. Blockage of the smooth muscle cell depolarisation through local stimulation of sympathetic neurons diminished peristalsis and it was reported that xysmalobinum, isolated from the roots, caused contraction of the smooth muscle of the uterus, intestine, bladder and bronchi (Watt, 1930; Schulzke et al., 2011). However, the mechanisms were poorly understood and Schulzke et al. (2011) therefore investigated the antisecretory effect of uzara. Secretory diarrhoea is characterised by the active secretion of chloride and/or bicarbonate into the intestine causing fluid loss; therefore developing new antisecretory agents is desirable. In addition, the introduction of oral rehydration therapy, which replaces water and electrolytes, has reduced the morbidity and mortality of diarrhoeal diseases. Epithelial secretory mechanisms were studied *in vitro* using HT-29/B6 cell lines (human colon carcinoma), which express features associated with a chloride secretory epithelium in addition to biopsy specimens from distal colon. These cells were stimulated using forskolin or cholera toxin. The cyclic adenosine monophosphate (cAMP)-dependent Cl^- secretion causes a lumen-negative voltage and a short-circuit current (I_{SC}) was measured. Tracer fluxes of $^{22}Na^+$ and $^{36}Cl^-$ were also measured. Two-path impedance spectroscopy was used to determine para- and transcellular resistance. A cAMP enzyme-linked immunosorbent assay (ELISA) kit was used to measure intracellular cAMP levels and the activity of Na^+/K^+-ATPase was monitored considering the blocking properties of ouabain. Uzara® Solution N manufactured by Stada in Germany with a concentration of 40 mg/ml glycosides in an alcoholic solution (43%) was purchased. In HT-29/B6 cells, uzara (50 μg/ml) inhibited the I_{SC} induced by the addition of forskolin (10 μM) or cholera-toxin (1 μg/ml) within 60 min implies reduced active chloride secretion. This result was comparable to the results obtained when human

colonic biopsies were pre-stimulated with forskolin. In addition, it was determined that the effect on forskolin-induced I_{SC} was time- and dose-dependent in HT-29/B6 cells. Forskolin also activates adenylate cyclase which causes an increase in the concentration of cAMP. Adenylate cyclase activates apical Cl^- channels required for efflux across the apical enterocyte membrane. Uzara partially blocked the increase in cAMP content caused by forskolin by almost 70%. Uzara inhibited Na^+/K^+-ATPase when added to the incubation medium of the cell monolayers ($37 \pm 6\%$) as well as when directly applied to the cells ($65 \pm 3\%$). Forskolin acts as a secretagogue causing Cl^- flux to markedly increase in the secretory direction. The addition of uzara prior to forskolin addition increased the flux in both directions but the mucosal-to-serosal flux was almost double. Uzara stimulated the Cl^- absorption and modulated the secretory Cl^- response to forskolin resulting in net Cl^- transport values of untreated monolayers. It was concluded that the antidiarrhoeal effects of uzara are due to the inhibition of active chloride secretion mainly as a result of Na^+/K^+-ATPase inhibition as well as a decrease in intracellular cAMP responses and paracellular resistance to a lesser extent. This study proved the antisecretory nature and mechanism of uzara and suggested that uzara could be useful in the treatment of secretory diarrhoea which is typically caused by bacterial pathogens such as *Escherichia coli* and *Vibrio cholera* (Schulzke et al., 2011).

6.2. Central nervous system activity

Depression is a serious disorder presenting with a diverse group of symptoms including psychological, physiological and behavioural. The pathophysiology of depression is intricate and it is believed that it is caused by a deficiency of neurotransmitters such as serotonin, noradrenalin and dopamine, although many other factors are involved. Selective serotonin reuptake inhibitors (SSRI), among the most frequently prescribed medicines, are used to treat depression by binding to a specific site on the SSRI site to inhibit the reuptake of serotonin. Uzara was included in a screening study to determine its affinity to the serotonin transporter (SERT). Water and ethanol extracts prepared from roots and leaves separately were tested using a serotonin reuptake transport protein-binding assay utilising a tissue suspension of whole rat brain (except the cerebellum). Concentrations of 5, 1, 0.1, 0.01 and 0.001 mg/ml were mixed with 50 μl of 4 nM [^{3}H]-citalopram and 50 μl of tissue suspension. The classification of activity was as follows: high affinity=concentration-dependent inhibition with less than 50% [^{3}H]-citalopram binding with the three strongest concentrations (5, 1 and 0.1 mg/ml); medium affinity=less than 50% [^{3}H]-citalopram binding in the two strongest concentrations (5 and 1 mg/ml); low affinity=less than 50% [^{3}H]-citalopram binding in the strongest concentration (5 mg/ml); no affinity=no concentration-dependent inhibition and the [^{3}H]-citalopram binding obtained between 70% and 130%. Uzara was classified in the high affinity to the serotonin transporter protein group: the aqueous extract of the aerial parts showed [^{3}H]-citalopram binding of 40% (5 mg/ml), 61% (1 mg/ml), 73% (0.1 mg/ml), 88% (0.01 mg/ml), 52% (0.001 mg/ml) and 20% (5 mg/ml), 31% (1 mg/ml), 47% (0.1 mg/ml), 73% (0.01 mg/ml) and 70% (0.001 mg/ml) for the ethanolic extract. The aqueous root extract exhibited binding of 51% (5 mg/ml), 68% (1 mg/ml), 81% (0.1 mg/ml), 117% (0.01 mg/ml) and 114% (0.001 mg/ml) while the ethanolic root extract showed binding of 76% (5 mg/ml), 93% (1 mg/ml), 107% (0.1 mg/ml), 82% (0.01 mg/ml) and 111% (0.001 mg/ml). At the three highest concentrations, uzara displaced more than 50% transport protein bound [^{3}H]-citalopram for the ethanolic leaf extracts. It was suggested that the clinical effects could be due to affinity for the serotonin receptor as it is known that the 5-HT 3 receptor is involved in the contraction of intestinal smooth muscle (Nielsen et al., 2004).

Pedersen et al. (2008) expanded on the study completed by Nielsen et al. (2004) by subjecting four plants selected based on the screening study to further testing. Ethanolic extracts were assayed for their affinity for the SERT, and their inhibitory effects on the SERT, the dopamine transporter (DAT) and the noradrenaline transporter (NAT) were determined. In addition, the antidepressant-like effects of the extracts were investigated using rat and mouse models. The [^{3}H]-citalopram binding assay revealed inhibition with an IC_{50} value of 1.1 ± 2.3 mg dry extract/ml confirming the results of the study by Nielsen et al. (2004). Using human SERT, NAT and DAT transfected in COS-7 (monkey kidney) cells, no significant SERT, DAT or NAT activity was recorded for the ethanolic uzara extract. No antidepressant-like effect was noted for uzara in the tail suspension test in mice where 125, 250 and 500 mg/kg of extract was administered by oral gavage 30 min before the 6-min test session commenced. The administration of 500 mg/kg of extract had no effect on the locomotor activity in rats and mice during the full 30-min test session. In the forced swim test in rats no anti-depressant effect was noted at the low dose (125 mg/kg) or high dose (500 mg/kg). However, in the forced swim test performed in mice ($n=9$ per group), doses of 250 and 500 mg/kg exhibited significant antidepressant-like activity, but not at 125 mg/kg. In the forced swim test, the immobility of mice is expressed as a percentage of the vehicle control and immobility is defined as the absence of movement except for movement needed to keep the head above water during the last 4 min of the 6 min period. The relative immobility was determined to be $77.6 \pm 6.0\%$ ($p < 0.05$) at a dose of 250 mg/kg and $67.9 \pm 8.2\%$ ($p < 0.01$) at 500 mg/kg compared to the controls imipramine (20 mg/kg) and desipramine (20 mg/kg) at 74.2 ± 3.7 and $54.4 \pm 4.8\%$ ($p < 0.001$), respectively. The inconsistent results between the rat and mouse tests highlight the importance of species comparisons to prevent false negative screening of compounds. Researchers noted that mice developed a distinct behavioural pattern after time had elapsed following uzara administration. Frequently rotating Straub tail, tics, tremor and hypolocomotion close to the ground was observed, indicating serotonin-like behaviour similar to that noted in SERT knockout mice. However, as no SERT, DAT or NAT activity was evident at the concentrations tested, another mechanism of action has to be responsible for this effect (Pedersen et al., 2008).

Alzheimer's disease, one of the most common neurodegenerative disorders causing dementia in elderly patients, is known to have a characteristic deficiency of acetylcholine (ACh). This neurotransmitter is hydrolysed by acetylcholine esterase (AChE) which, when inhibited may lead to an improvement of the cognitive function in these patients. An *in vitro* study for AChE inhibition was performed using Ellman's colourimetric method on the ethyl acetate and methanol extracts (7, 16, 31, 63 and 125 ng/ml) of 12 medicinal plants including uzara (Adewusi and Steenkamp, 2011). The ethyl acetate extract of uzara root at a concentration of 125 ng/ml showed AChE inhibition with an IC_{50} value of 0.5 ng/ml (galanthamine control $IC_{50}=0.053$ ng/ml). As reactive oxygen species (ROS) has been implicated in the pathogenesis of Alzheimer's disease, the anti-oxidant activity was also determined. However, neither the ethyl acetate nor the methanol extract showed anti-oxidant activity (> 50%) in the 2,2-diphenyl-1-picrylhydrazyl 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays at the highest concentration tested (0.125 mg/ml).

The monoamine oxidase (MAO) enzyme is present in the outer mitochondrial membrane of neuronal and non-neuronal cells and the two isoforms, MAO-A and MAO-B, have different functions. MAO-A inhibitors are used to treat depression and anxiety

disorder and MAO-B inhibitors are employed in the treatment of Parkinson's and Alzheimer's disease as it increases the basal dopamine levels (Stafford et al., 2007). Stafford et al. (2007) determined the *in vitro* MAO inhibitory activity of water, 70% ethanol, ethyl acetate and petroleum ether extracts of 20 plants at concentrations of 1, 0.5, 0.25, 0.1, 0.01, 0.001 and 0.0001 mg/ml in phosphate buffer using the continuous peroxidase-linked photometric assay and MAO isolated from rat liver homogenates. Very weak non-selective MAO inhibition with an IC_{50} value of 849 ± 110 μg/ml was noted for the ethyl acetate extract of uzara root (Stafford et al., 2007).

South African plants such as uzara are often employed as sedatives or to treat various CNS-related ailments or mental diseases. GABA or γ-aminobutyric acid is an inhibitory neurotransmitter which exerts its effect by binding to the GABA receptors (A,B,C) in the neuronal membrane. Compounds such as benzodiazepines which have anxiolytic, anticonvulsant, sedative-hypnotic and muscle relaxant effects bind to the $GABA_A$ receptor to modify the chloride channel gating. In a screening study, 46 ethanol extracts from 35 species used traditionally as sedatives or to treat mental disorders were tested using the $GABA_A$ benzodiazepine receptor-binding assay. Ethanolic root and leaf extracts (0.456, 0.046, 0.005 mg/ml) from uzara were tested using rat cerebral cortices in the ^{3}H-Ro 15-1788 or flumazenil (benzodiazepine agonist)-binding assay where diazepam was used to determine non-specific binding and specific binding was calculated by subtracting non-specific binding from the total binding. The percentage binding of Ro 15-1788 to the $GABA_A$-benzodiazepine receptor in the presence of the extracts was as follows; leaf extracts (0.456, 0.046, 0.005 mg/ml): $52.5 \pm 2.6\%$, $80.2 \pm 1.1\%$, $84.4 \pm 0.8\%$; and root extracts (0.456, 0.046, 0.005 mg/ml): root $72.6 \pm 5.1\%$, $84.9 \pm 0.9\%$, $86.2 \pm 4.5\%$. Although uzara exhibited very weak activity in this assay, a different mechanism may be responsible for the effects (Stafford et al., 2005).

6.3. Screening studies

Uzara has not been the principal focus in many scientific investigations but it has been incorporated as one of the plants in the screening panel of several studies. Results which have not been discussed in the preceding sections are described below.

6.3.1. Antiplasmodial activity

In a screening study to determine the *in vitro* antiplasmodial activity of medicinal plants growing in South Africa, 134 plant taxa representing 54 families selected using weighted criteria were tested against *Plasmodium falciparum* strain D10 (chloroquine-sensitive). Serial dilutions were done to achieve a concentration range of 100–0.2 μg/ml of extract in 96-well microtitre plates and IC_{50} values were obtained from the dose–response curve. For this study, an IC_{50} value of ≤ 10 μg/ml was considered as promising activity and a value of ≤ 5 μg/ml was classified as highly active. Inhibition of parasite growth at low concentrations would indicate selective activity rather than non-specific activity as is often noted at high concentrations. A whole plant extract of uzara prepared using dichloromethane/methanol (1:1) exhibited promising activity with an IC_{50} value of 6 μg/ml (Clarkson et al., 2004). However, the reportedly promising antiplasmodial effect has not been further explored.

6.3.2. Antibacterial and antifungal activity

Many plants or plant extracts have been used to treat infectious diseases throughout history and they are very commonly investigated to determine their antimicrobial activity. Venereal disease (VD), also known as sexually transmitted diseases (STDs), is considered to be particularly responsive to treatment with traditional medicines. Buwa and Van Staden (2006) screened the aqueous and ethanolic extracts of 13 medicinal plants used by South African traditional healers for the treatment of venereal disease for antibacterial and antifungal activity. The minimal inhibitory concentration (MIC) method was used to determine the activity of these extracts at concentrations of 12.5 mg/ml to 0.38 μg/ml against *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Klebsiella pneumoniae* (ATCC 13883) and *Staphylcoccus aureus* (ATCC 12600) using neomycin as a reference standard as well as *Candida albicans* (ATCC 10231) with Amphotericin B as a reference standard. Antibacterial activity was determined using the visual result obtained when 40 μl of *p*-iodonitrotetrazolium violet (INT) was added to the wells while the antifungal results were determined using absorbance at 630 nm in an ELISA reader. The uzara water extract exhibited an MIC value of 12.5 mg/ml against three bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Staphylcoccus aureus*) and *Candida albicans*. No effect was observed against *Bacillus subtilis* at the maximum concentration tested (12.5 mg/ml). The MIC recorded for the uzara ethanol extract against the four bacteria was 3.125 mg/ml (Buwa and Van Staden, 2006). Uzara exhibited limited value as an antimicrobial agent against the examined pathogens.

6.3.3. Wound-healing

Many plants, including uzara, are purportedly used in wound-healing. The complex process of wound healing includes homoeostasis, re-epithelialisation, granulation tissue formation and remodelling of the extracellular matrix. It is said that the powdered tuber of uzara is applied to sores, wounds and abscesses. Aqueous and methanolic root extracts were prepared and its antibacterial effects were investigated as a first step. The uzara extracts exhibited MIC values of > 4 mg/ml against *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (clinical isolate), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 278930). This result supported various studies indicating that uzara does not exhibit antimicrobial activity. As a result of this, the authors excluded uzara from the screening panel in further studies where the anti-oxidant activity of the extracts was assessed by measuring the oxidant generation by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated human neutrophils as well as the effect on fibroblast growth stimulation in human fibroblasts (Steenkamp et al., 2004). As reported in Section 6.2, the ethyl acetate and methanol extracts of uzara did not exhibit anti-oxidant activity in the DPPH and ABTS assays at 0.125 mg/ml (Adewusi and Steenkamp, 2011).

6.3.4. Mutagenic and antimutagenic effects

Herbal medicines are generally assumed to be safe for use with no side-effects due to their long traditional use. However, it is most important to evaluate all plants for safety as many cases of toxicity have been recorded. Possible gene mutations can be determined using the *Salmonella*/microsome mutagenicity assay (Ames). In this study, dichloromethane was used to prepare a leaf extract of uzara (extract 1) which was further extracted with 90% methanol (extract 2) – in total 42 South African plants were screened. Extracts were dissolved in 10% dimethylsulphoxide (DMSO) to concentrations of 5.0, 0.5 and 0.05 mg/ml for mutagenic screening using *Salmonella typhimurium* (TA98 and TA100). An extract is considered mutagenic when the mean number of revertants was at least double that found in the solvent control culture (10% DMSO). A variation of the test is used for antimutagenic screening where the percentage inhibition was calculated; non-mutagenic effect = value $< 25\%$; moderate antimutagenicity = value of 25–40%; and strong antimutagenicity = value of

> 40%. None of the dichloromethane extract exhibited mutagenic activity. None of the uzara leaf extracts exhibited mutagenic or antimutagenic effects (Reid et al., 2006).

6.4. Dysmenorrhoea

The use of uzara to treat menstrual and afterbirth cramps (dysmenorrhoea) and related symptoms, based on the ethnopharmacological use, has not been scientifically proven, except for the general effect uzara was reported to have on smooth muscle in the 1930s and the suppositions made as a result of these studies. However, an interventional Phase III prospective randomised comparative two-way cross-over assignment safety/efficacy pilot study to test the efficacy of uzara in primary dysmenorrhoea was registered (ISRCTN25618258) in Egypt in 2011. The main aim was to test the efficacy of uzara in dysmenorrhoea and the secondary outcomes will be to monitor for adverse events and drug tolerability. No further information on this trial is available and it is not certain whether this trial is going ahead as planned but this information was last updated in August 2014 and it seems that recruitment ($n=60$) has been completed. More information can be found on the World Health Organisation International Clinical Trials Registry Platform (World Health Organisation (WHO), 2014).

7. Pharmacokinetic and pharmacodynamic evaluation

Schmiedl et al. (2012) performed cross-reactivity studies of uzara compounds with digitoxin and digoxin (described in Section 8) and at the same time determined the pharmacokinetic and pharmacodynamic parameters in 18 healthy volunteers after the oral administration of a commercial product, Uzara® Losüng N. The label of this product states that the preparation is an oral solution containing 40 mg/ml of uzara; it may only be sold in a pharmacy; and that the active ingredient is the dry extract of Uzarae radix (root). This study was a single-blind, randomised, placebo and verum-controlled design with 3 cross-over periods employing the double-dummy technique. The following treatments were administered to each subject: 1) a single oral dose of 5 ml Uzara® Losüng N (=200 mg root extract or 75 mg uzara glycosides) plus intravenous (i.v.) placebo; 2) oral placebo plus a single 1.0 mg (4 ml) intravenous dose of digoxin (Lanicor®); 3) oral plus i.v. placebo. All examinations were performed after the subjects had been in a supine position for at least 30 min. For pharmacokinetic studies, blood samples were obtained at the following intervals: 0:15 h pre-dose and 0:15, 0:45, 1:15, 2:00, 3:00, 4:00, 5:00, 6:00, 8:00, 10:00, 15:00, 24:00 and 26:00 h post-dose; urine samples were collected until 36:00 h post-dose at 4 intervals. Due to levels of uzarin, uzarigenin and xysmalorin below the quantification limit as determined by HPLC-MS/MS, pharmacokinetic analysis could only be performed for *allo*uzarigenin. Marked inter-individual variability was noted and the median values as well as the minimum and maximum values were calculated: C_{max} (peak plasma concentration)=0.39 ng/ml (Min=0.15; Max=1.81); t_{max} (time to reach C_{max})=7.0 h (Min=3.0; Max=36.0); $T_{½}$ (half-life)=5.2 h (Min=0.8; Max=23.6); $AUC_{0-36\ h}$ (area under the curve, integral of the concentration–time curve)=4.2 ng/ml h (Min=0.8; Max=11.1) and $AUC_{0-\infty\ h}$=5.8 ng/ml h (Min=1.8; Max=13.1). No significant cardiovascular pharmacodynamic changes were caused by uzara in this study. Digoxin (positive control) reduced diastolic blood pressure, shortened systolic time intervals, exhibited positive inotropic effects, reduced heart rate and altered electrocardiogram (ECG) parameters significantly as expected (Schmiedl et al., 2012).

8. Toxicity and interference with laboratory tests

Watt (1930) reported that isolated xysmalobinum is toxic and produces a digitalis-like action on the heart but that the degree of toxicity is low compared to other glucosides and less than that of a dry alcoholic extract of the root. The contraction of the smooth muscle of the bronchi caused by xysmalobinum plays a role in the purported toxicity due to respiratory distress (Watt, 1930). Brown et al. (1983) determined the positive inotropic potency of uzarigenin, uzarigenin rhamnoside, uzarigenin glucoside, uzarigenin diglucoside (uzarin), and uzarigenin triglucoside (uzaroside). The equilibrium dissociation constant (K_D) for the binding of these compounds to guinea pig heart Na^+/K^+-ATPase, determined using [^{3}H]-ouabain displacement, was calculated to be 1550, 69, 1450, 4330 and 2460×10^{-9} M. The potency relative to digitoxigenin (1.0) was determined to be 0.20, 4.50, 0.21, 0.07 and 0.13. The ΔF_{75} values, defined as the drug concentration effecting a 75% increase in force of contraction as interpolated from cumulative dose–response curves, were 1.2, 9.3, 0.44, 0.12 and 0.22, indicating that uzara administration can have digitalis-like effects on the heart (Brown et al., 1983; Schulzke et al., 2011). However, as mentioned in Section 5.4, the uzara cardenolides and glycosides have little cardiotonic activity. In addition, Schmelzer (2011) noted that poisoning is unlikely when uzara is orally consumed as the glycosides are poorly absorbed.

Uzara root is named in a review of abnormal laboratory test results and toxic effects due to the use of herbal medicines as an agent that interferes with digoxin assays and has an additive effect with digoxin due to the presence of cardenolide glycosides (Dasgupta, 2003). Digoxin has a narrow therapeutic index (0.8–1.8 ng/ml) and is one of the most frequently named medicines in clinically significant adverse drug–drug interaction cases. Therefore, blood levels need to be carefully regulated. The interference of uzara roots with digoxin is classified in the “significant cross-reactivity” category and it also shows additive effects if digoxin is present in the serum of the patient (Dasgupta, 2011). In a case report, Thürmann et al. (2004) presented the case of a 68-year old woman who was admitted to the emergency department with acute respiratory distress and pulmonary oedema, depressed left ventricular function and tachyarrhythmia absoluta was noted. As part of her treatment, a loading dose (0.75 mg) and second dose (0.1 mg) of digitoxin were administered. Her serum digitoxin concentration was determined to be > 100 μg/l using the CEDIA digitoxin test (Roche Diagnostics GmbH, Mannheim, Germany), while the therapeutic range is 10–25 μg/ml. Although no symptoms of digitalis toxicity was noted, treatment was withheld until therapeutic concentrations were achieved after 4 days. Further investigation revealed that the patient had taken three 30 ml (15 mg glycosides/ml) doses of uzara 2 days before she presented to the emergency department. The assumption of interference between uzara glycosides and digitoxin in the assay was further evaluated in a pilot study on 4 healthy volunteers where 30 drops of uzara (about 1.5 ml ≅ 22 mg glycosides) were administered and blood levels were monitored for 32 h. In one volunteer, digoxin was monitored for 24 h. Interference with the assay kits was recorded after the administration of a single dose, which was less than the manufacturers recommendations. Serum levels of digitoxin and digoxin were determined to be above therapeutic levels using the digitoxin (CEDIA digitoxin test (Roche Diagnostics GmbH, Mannheim, Germany) and digoxin assays (Tina-quant digoxin test, Roche Diagnostics GmbH, Mannheim, Germany). The authors concluded that the half-life in healthy volunteers was approximately 9 h and that the influence of uzara on therapeutic drug monitoring (TDM) disappeared after 2–3 days. It was also suggested that the cause of hospitalisation could have been due to uzara and that a cardiotropic effect at very high doses

cannot be ruled out. The manufacturers' listed contraindication in patients with heart failure requiring digitalis therapy should be followed and the authors suggested that it should be extended to patients with a high risk of developing heart failure and/or arrhythmia (Thürmann et al., 2004.

Schmiedl et al. (2012) further investigated the cross-reactivity phenomenon after the administration of a single 5 ml oral dose of Uzara® Liquid N (75 mg uzara glycosides) to 18 healthy volunteers as described in Section 7. The positive control, digoxin (1 mg i.v.) significantly altered cardiovascular pharmacodynamic parameters but the administration of the recommended single dose of uzara extract did not. Considerable digitalis-like serum levels (DLSL) could be detected with extremely high cross-reactivity of uzara compounds with the commercially available conventional digitalis assays tested which included: Cobas Integra® (Roche Diagnostics) and Tina-quant® (Roche Diagnostics) digoxin assays, as well as Online TDM® (Roche Diagnostics) and Cobas Integra® (Roche Diagnostics) digitoxin assays. Safety evaluations performed revealed 28 adverse events of which 24 were classified as mild and 4 as moderate, reported by 13 subjects (11 in the digoxin group, 10 in the placebo group, 6 in the uzara group and 1 before administration). Headache and fatigue were reported in most cases but all adverse events resolved completely and no indications for adverse events were noted in laboratory results. The authors noted that their results cannot exclude cardiovascular effects in patients with pre-existing cardiac disease, especially at higher than recommended doses of uzara. In addition, it is imperative for physicians to ensure that they enquire about any complementary medicines patients may be using (Schmiedl et al., 2012).

9. Commercial aspects

Uzara has been used to treat diarrhoea for more than 100 years and was introduced into the pharmaceutical market in Germany in 1911 as a "new intestinal sedative" (antidiarrhoicum). In 1927, a tablet prepared from the root containing amongst others the active compound, uzaron (0.05 g) and diamethylaminophenazon (0.30 g), was introduced into the German market under the name Dysmenural®; however, this product is not manufactured anymore. Extracts of the roots have been marketed in Europe as "uzarae radix" to treat acute diarrhoea and menstrual cramps. It is said that the daily dose should not exceed 90 mg total glycosides, calculated as uzarin (Schmelzer, 2011; Schulzke et al., 2011).

Dold and Cocks (2002) surveyed the trade in medicinal plants in the Eastern Cape Province. They aimed to document the species traded, determine the quantities harvested annually and to assess the economic value of trade. All the trade role players were included; from informal street hawkers to herbal medicine consumers and a total of 282 questionnaires were completed. Uzara featured at position number 51 on the list of the top 60 most frequently traded plants but specific trade information could not be determined. *Xysmalobium* species collectively was ranked at number 54 and it was estimated that 63.3 kg per trader was traded annually calculated from 5 respondents. Unfortunately, the mean price per kilogram could not be determined for *Xysmalobium* species (Dold and Cocks, 2002). According to Foden and Potter (2005), *Xysmalobium undulatum* is classified as "least concern" (LC) on the red list of plants of South Africa as it was not included in the threatened species programme. LC species are considered at low risk of extinction and widespread and abundant species are typically classified in this category. However, according to Dold and Cocks (2002), it is a protected species based on the Cape Nature and Environmental Conservation Ordinance. Demands for large quantities of root material may have a profound effect on the availability of the species as well as the price per kilogram. In addition, the process is unsustainable as the harvesting of roots causes death of the plant.

In contrast with many other well-known natural products such as Devil's claw or *Harpagophytum procumbens* (Burch.) DC. ex Meisn. subsp. *procumbens*, very few patents have been filed for uzara. Using a Google® patent search, 1210 results were returned for *Harpagophytum procumbens* compared to 8 when using *Xysmalobium undulatum* as search words. These include "Water-soluble formulations of digitalis glycosides for treating cell-proliferative and other diseases", Topical and oral formulations of cardiac glycosides for treating skin diseases" and "Medicaments containing plant extracts" and most of these were filed in duplicate or triplicate (United States patent, World Intellectual Property Organisation patent and European patent). Although Van Wyk (2011) identified *Xysmalobium undulatum* as one of the plants earmarked for further commercialisation, this has not occurred, despite the fact that the available scientific evidence supports its traditional use.

10. Conclusions

Uzara has been used traditionally and as an antidiarrhoeal in Europe for more than 100 years. In contrast to many other traditional medicines, its phytochemistry has been most intensively investigated. Despite the findings of these studies, such as that very little cardiotonic effect is evident, further commercial development of uzara is not apparent. It is hypothesised that this could be due to the presence of cardenolide glycosides which may have a digitalis-like action on the heart, toxicity is therefore assumed, and these results have not been sufficiently disputed. In addition, its acceptability by the consumer may be limited due to its very bitter taste. Most recently, uzara was found to not significantly affect selected pharmacodynamic parameters (Schmiedl et al., 2012). Schulzke et al. (2011) showed that the antidiarrhoeal effect of uzara was due to an antisecretory mechanism of action. This is a significant finding as the search for agents that will directly inhibit intestinal secretory mechanisms has continued for more than 20 years (Farthing, 2006). Most of the scientific studies have been of a screening nature where uzara was included as one of the most commonly used medicinal plants in South Africa. No clinical studies to assess the therapeutic efficacy of uzara in the treatment of diarrhoea have been performed to date. Many of the effects reported for uzara dates back to the early 1900s and it seems appropriate to reconfirm these effects, perform the appropriate toxicity studies and move towards clinical testing.

Although an impressive volume of phytochemical research has been done on uzara, there remains an opportunity to further unravel the structure-activity relationships of uzara glycosides and to determine and differentiate the pharmacophores for the spasmolytic and cardiotonic effects. It has been noted that there is a need for new inotropic medicines as it is clear that the medicines currently available are inadequate to restore health as well as the prevalence of congestive heart failure. The search for non-steroidal digitalis alternatives has not been successful and the search has shifted back towards ideal inotropic steroids (Repke et al., 1996). From this review it is evident that there is a need for further research on uzara as many promising leads have been identified and a solid basis exists to convert this knowledge and scientific information into evidence-based phytomedicines.

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